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Short Communication

Frequent *FGFR3* Mutations in Papillary Non-Invasive Bladder (pTa) Tumors

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We recently identified activating mutations of fibroblast growth factor receptor 3 (FGFR3) in bladder carcinoma. In this study we assessed the incidence of *FGFR3* mutations in a series of 132 bladder carcinomas: 20 carcinoma *in situ* (CIS), 50 pTa, 19 pT1, and 43 pT2–4. All 48 mutations identified were identical to the germinal activating mutations that cause thanatophoric dysplasia, a lethal form of dwarfism. The S249C mutation, found in 33 of the 48 mutated tumors, was the most common. The frequency of mutations was higher in pTa tumors (37 of 50, 74%) than in CIS (0 of 20, 0%; $P < 0.0001$), pT1 (4 of 19, 21%; $P < 0.0001$) and pT2–4 tumors (7 of 43, 16%; $P < 0.0001$). *FGFR3* mutations were detected in 27 of 32 (84%) G1, 16 of 29 (55%) G2, and 5 of 71 (7%) G3 tumors. This association between *FGFR3* mutations and low grade was highly significant ($P < 0.0001$). *FGFR3* is the first gene found to be mutated at a high frequency in pTa tumors. The absence of *FGFR3* mutations in CIS and the low frequency of *FGFR3* mutations in pT1 and pT2–4 tumors are consistent with the model of bladder tumor progression in which the most common precursor of pT1 and pT2–4 tumors is CIS. *(Am J Pathol 2001, 158:1955–1959)*

Bladder cancer is the fourth most common malignancy in men and the ninth most common in women in the Western world. In these countries, more than 90% of bladder tumors are urothelial carcinomas. At the time of initial diagnosis, approximately 80% of urothelial carcinomas are confined to the epithelium (pTa, CIS) or lamina propria (pT1), whereas the remaining 20% invade the muscularis propria (pT2, pT3, pT4). pTa lesions, the most common form of bladder carcinoma, are papillary tumors. Carcinoma *in situ* (CIS) are flat, cytologically high-grade carcinomas. Primary isolated CIS is a very rare entity, CIS being more commonly associated with other malignant bladder lesions.[1]

Clinical evidence and molecular studies suggest that there are two pathways in bladder carcinogenesis responsible for generating two types of urothelium-confined tumors (pTa and CIS) with very different behavior.[1–6] pTa tumors are associated with a high rate of recurrence (50–75%) but a low probability (<5%) of progression to lamina propria-invasive (pT1) and muscle-invasive (pT2–4) tumors. CIS may be the most common precursor of invasive bladder cancer because CIS shows a strong tendency to progress (40–50%), and because most muscle-invasive lesions arise with no history of a pTa precursor lesion. This clinical evidence is supported by various molecular studies showing that CIS and invasive tumors have many genetic alterations in common, such as specific chromosomal deletions and a high frequency of p53 mutations.[2,7]

In our search for new markers of carcinoma progression, we recently reported specific missense mutations in a gene encoding a growth factor receptor, fibroblast

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growth factor receptor 3 (FGFR3), in a series of 26 bladder and 12 cervical tumors.[8] FGFR3 belongs to a family of structurally related tyrosine kinase receptors encoded by four different genes (*FGFR1–4*). These receptors are glycoproteins composed of two or three extracellular immunoglobulin (Ig)-like domains, a hydrophobic transmembrane domain, and a cytoplasmic region that contains the tyrosine kinase domain. Binding to members of the fibroblast growth factor family induces FGFR dimerization, resulting in autophosphorylation of the kinase domain and interaction with and phosphorylation of effector signaling proteins. Alternative mRNA splicing mechanisms generate many different receptor isoforms.[9] Isoforms FGFR3b and FGFR3c result from a mutually exclusive splicing event in which the second half of the juxtamembrane Ig-like domain of FGFR3 is encoded by either the 150 nucleotides of exon 8 or the 144 nucleotides of exon 9.[10] FGFR3b and FGFR3c have different tissue distributions; for example, FGFR3b is the main form in epithelial cells, whereas FGFR3c is the form found in chondrocytes.[11] Specific point mutations in various domains of FGFR3 are associated with autosomal dominant human skeletal disorders such as hypochondroplasia, achondroplasia, severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), and thanatophoric dysplasia.[12,13] Several reports have demonstrated that these mutations lead to constitutive activation of the receptor.[12–14] The identification in multiple myeloma and, more recently, in bladder and cervical carcinomas of somatic mutations of *FGFR3* identical to the activating mutations responsible for thanatophoric dysplasia and SADDAN[8,15–17] suggested that FGFR3 plays an oncogenic role.

We investigated the involvement of *FGFR3* in bladder carcinogenesis by assessing the incidence of *FGFR3* mutations in a series of 132 bladder tumors of various stages and grades. The frequency of *FGFR3* mutations (74%) in non-invasive papillary pTa tumors shows that FGFR3 is a major oncogene in this, the most common form of bladder carcinoma. This high frequency of *FGFR3* mutations in pTa tumors contrasts with the absence of *FGFR3* mutations in carcinoma *in situ* and the low percentage of mutations in pT1 and pT2–4 tumors. These data are consistent with the existence of two pathways of progression in bladder cancer.

Materials and Methods

Tumor Samples

Tumors were staged according to the TNM classification[18] and graded according to the criteria recommended by the World Health Organization.[19] All slides were reviewed by two pathologists (CB, MPB). For cases in which lamina propria invasion was unclear, we performed immunohistochemical analysis with an anti-cytokeratin antibody. The bladder tumor series consisted of 20 carcinoma *in situ* (CIS), 50 pTa, 19 pT1, 14 pT2, 20 pT3, and 9 pT4 tumors; 32 were grade G1, 29 grade G2, and 71 grade G3. The series of 112 pTa and pT1–4 tumors were obtained from 112 patients. The 20 CIS were obtained from an additional 17 patients. Nine CIS occurred as isolated lesions; eleven were associated with pTa or pT1–4 tumors. All of the pTa and pT1–4 tumors and 10 of the CIS were obtained from patients diagnosed at the Henri Mondor Hospital (Créteil, France). The 10 remaining CIS were obtained from patients diagnosed at the Rotterdam University Hospital (Rotterdam, The Netherlands). DNA from pTa and pT1–4 tumors was extracted from samples frozen immediately in liquid nitrogen as previously described[20] or from formalin-fixed, paraffin-embedded tissue, using the tissue DNA extraction kit from Qiagen (Courtaboeuf, France). All samples retained for this analysis contained more than 80% tumor cells, as assessed by histological examination. For CIS, tumor cells were isolated from paraffin-embedded sections by laser-assisted microdissection, using the PALM Laser-Microbeam System (PALM, Wolfratshaussen, Germany), and DNA was extracted using the Qiagen kit. Normal DNA samples from peripheral blood were available for 45 patients.

FGFR3 Mutation Analysis

Mutations were screened by single-strand conformation polymorphism in four regions of the *FGFR3* gene, located in exons 7, 10, 15, and 19. These four regions contain the *FGFR3* point mutations previously identified in thanatophoric dysplasia, SADDAN, achondroplasia, Crouzon syndrome with acanthosis nigricans, multiple myeloma, and bladder and cervical carcinomas.[8,12,13]

For most of the samples (all pTa and pT1–4 tumors and 16 CIS), a single round of PCR was carried out, using the following primer pairs: exon 7, 5′-AGTGGCGGTGGTGGTGAGGGAG-3′ and 5′-TGTGCGTCACTGTACACCTTGCAG-3′; exon 10, 5′-CAACGCCCATGTCTTTGCAG-3′ and 5′-CGGGAAGCGGGAGATCTTG-3′; exon 15, 5′-GACCGAGGACAACGTGATG-3′ and 5′-GTGTGGGAAGGCGGTGTTG-3′; exon 19, 5′-TGTCGGCGCCTTTCGAGCAGTA-3′ and 5′-AGCAGCAGGGTGGGCTGCTA-3′. PCR was performed in a final volume of 50 μl containing 50 ng genomic DNA or 1/20th of the purified DNA from microdissected samples, 100 μmol/L each of dATP, dCTP, dTTP, and dGTP, 1 μmol/L forward and reverse primers, 1× of amplification buffer provided with the polymerase and 1 μCi [α^{33}P]dATP. The *Taq* polymerase (1 U; Finnzymes Oy, Espoo, Finland) was added after heating the reaction mixture for 5 minutes at 95°C. The mixture was then subjected to 35 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute, 20 seconds.

A two-stage seminested PCR was used to amplify DNA from 4 CIS that gave a weak signal in single-round PCR. For the first round of seminested PCR we used the following PCR primers: exon 7, 5′-AGTGGCGGTGGTGGTGAGGGAG-3′ and 5′-TGTGCGTCACTGTACACCTT-3′; exon 10, 5′-GCCAGGCCAGGCCTCAAC-3′ and 5′-CTTGAGCGGGAAGCGGGAGATCTTG-3′; exon 15, 5′-TGGTGACCGAGGACAACGTGATG-3′ and 5′-CTCTGGTGAGTGTAGACTCG-3′. For the second round of seminested we used the following PCR primers: for exon 7, 5′-AGTGGCG-

Table 1. Point Mutations in the *FGFR3* Gene in Bladder Carcinomas

Codon	nt Position	Exon	Mutation	Predicted effect	Number of tumors
248	742	7	CGC→TGC	Arg→Cys	8
249	746	7	TCC→TGC	Ser→Cys	33
372 (370)	1,114	10	GGC→TGC	Gly→Cys	3
375 (373)	1,124	10	TAT→TGT	Tyr→Cys	2
652 (650)	1,954	15	AAG→GAG	Lys→Glu	2

Codon and mutated nucleotide (nt position) are numbered according to the cDNA open reading frame corresponding to the FGFR3b isoform. This isoform is produced in epithelial cells and contains two more amino acids than the FGFR3c isoform present in bones. The codon number according to the FGFR3c cDNA open reading frame is indicated in parentheses.

GTGGTGGTGAGGGAG-3′ and 5′-TGTGCGTCACTGTACACCTTGCAG-3′; exon 10 5′-CCTCAACGCCCATGTCTTTTCAGC-3′ and 5′-CTTGAGCGGGAAGCGGGAGATCTTG-3′; exon 15, 5′-TGGTGACCGAGGACAACGTGATG-3′ and 5′-AGGGTGTGGGAAGGCGGTGTTG-3′. First-round PCR conditions were the same as for single-round PCR, except that no radioactive isotope was included in the reaction and only 15 cycles were performed. The first-round PCR product (1 μl) was subjected to a second round of PCR comprising 35 cycles with conditions identical to those for single-round PCR except that the annealing temperature was 68°C.

Aliquots of the labeled PCR products were subjected to electrophoresis in non-denaturing mutation detection enhancement (MDE)/8.5% glycerol gels at room temperature for 13 hours. Samples displaying a mobility shift in single-strand conformation polymorphism analysis were further analyzed by direct bidirectional DNA sequencing. The PCR products were sequenced using the following primers: exon 7, 5′-AGTGGCGGTGGTGGTGAGGGAG-3′ and 5′-CAGCACCGCCGTCTGGTTGG-3′; exon 10, 5′-CAACGCCCATGTCTTTGCAG-3′ and 5′-GAGCCCAGGCCTTTCTTGG-3′; exon 15, 5′-AGGACAACGTGATGAAGATCG-3′ and 5′-GTGTGGGAAGGCGGTGTTG-3′; exon 19, 5′-TGTCGGCGCCTTTCGAGCAGTA-3′ and 5′-TGCTAGGGACCCCTCACATT-3′. Matched normal DNA, if available, was sequenced on both strands to demonstrate the somatic nature of these mutations.

Results

Identification of FGFR3 Point Mutations

We used single-strand conformation polymorphism and sequencing to analyze the regions of *FGFR3* harboring the point mutations found in multiple myelomas and in skeletal disorders, in a series of 132 bladder carcinoma samples. We detected five different single-nucleotide substitutions in 48 of the 132 bladder carcinomas (Table 1). These mutations affected codons 248, 249, 372, 375, and 652 (FGFR3b isoform numbering). Four of these five mutations were located in the extracellular domain (codons 248 and 249) or transmembrane domain (codons 372 and 375), with the mutated codon encoding a cysteine residue in each case. The fifth mutation, located in the kinase domain (codon 652), resulted in the replacement of a positively charged residue by a negatively charged residue. The S249C mutation was the most frequent and was found in 33 of the 48 mutated tumors (69%). The Y375C mutation is reported here for the first time in bladder tumors. All five mutations identified in bladder carcinomas were identical to the germinal activating mutations responsible for thanatophoric dysplasia, a lethal form of dwarfism. As expected, matched constitutional DNA, available for 15 cases with mutations, contained wild-type sequences demonstrating the somatic nature of these mutations.

Relationship between FGFR3 Mutation Status, Tumor Stage, and Histological Grade

We assessed the frequency of *FGFR3* mutations in the two types of non-invasive bladder cancer (CIS and pTa tumors), and in lamina propria-invasive (pT1) and muscle-invasive (pT2–4) tumors. The distribution of *FGFR3* mutations as a function of stage is shown in Figure 1a. *FGFR3* mutations were observed in 37 of 50 pTa tumors (74%), whereas no mutation was detected in the 20 CIS examined. This difference in mutation frequency between these two groups of tumors was highly significant ($P < 0.0001$; two-sided P, Fisher's exact test). *FGFR3* mutations were detected in 4 of 19 (21%) pT1 tumors and in 7 of 43 (16%) pT2–4 tumors. The difference in frequency of *FGFR3* mutations between pTa and pT1 tumors was highly significant ($P < 0.0001$). The difference in frequency of *FGFR3* mutations between CIS and pT1 was at the limit of significance ($P = 0.05$). In contrast, there was clearly no difference in the frequency of *FGFR3* mutations between pT1 and pT2–4 tumors ($P = 0.7$).

The distribution of *FGFR3* mutations according to histological grade is shown in Figure 1b. *FGFR3* mutations were detected in 27 of 32 (84%) G1 tumors, 16 of 29 (55%) G2 tumors but in only 5 of 71 (7%) G3 tumors. This

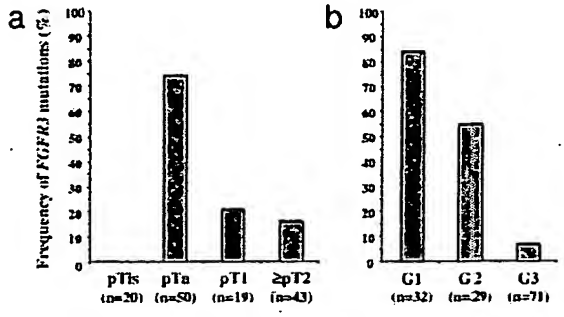

Figure 1. *FGFR3* mutations according to stage or grade. a: Percentage of *FGFR3* mutations in carcinoma *in situ*, pTa, pT1, and pT2–4 tumors. b: Percentage of mutations as a function of tumor grade.

Table 2. *FGFR3* Mutations According to Grade in pTa, pT1, and pT2–4 Tumors

Stage	Grade	*FGFR3* wild-type	*FGFR3* mutant	*P* value*
pTa**	G1	5	27 (84%)	0.013
	G2	4	9 (69%)	
	G3	4	1 (20%)	
pT1	G1	0	0	0.097
	G2	1	2 (67%)	
	G3	14	2 (12.5%)	
≥pT2	G1	0	0	0.019
	G2	8	5 (38%)	
	G3	28	2 (7%)	

For each grade, the percentage of tumors with mutations in *FGFR3* is indicated.
*Comparison G1 + G2 *versus* G3, two-sided *P*, Fisher's exact test.
**According to the consensus classification of 1998,[21] the pTaG1 + pTaG2 group of tumors corresponds to papillary neoplasms of low malignant potential + low-grade papillary carcinomas, whereas pTaG3 tumors correspond to high-grade papillary carcinomas.

association between *FGFR3* mutations and low grade was highly significant ($P < 0.0001$; two-sided *P*, χ^2 test). Table 2 shows the distribution of *FGFR3* mutations as a function of grade for the three groups of tumors classified according to stage (pTa, pT1, and ≥ pT2 tumors). A highly significant correlation was found between *FGFR3* mutations and low grade (G1 + G2 *versus* G3) within the pTa tumor group ($P = 0.009$; two-sided *P*, Fisher's exact test) and within the ≥pT2 tumor group ($P = 0.02$; two-sided *P*, Fisher's exact test). The correlation between *FGFR3* mutations and low grade was not significant for pT1 tumors, probably due to the small number of samples in this group.

Discussion

Germinal point mutations resulting in FGFR3 activation are responsible for dwarfism syndromes. Surprisingly, similar FGFR3 mutations have also been implicated in tumorigenesis. Somatic FGFR3 mutations identical to those found in thanatophoric dysplasia (neonatal lethal dwarfism syndrome) and SADDAN have been associated with rare cases of human multiple myeloma.[12,13] We recently identified, in a series of 26 bladder and 12 cervix carcinomas, several FGFR3-activating mutations previously identified as associated with thanatophoric dysplasia.[8] To investigate further the role of *FGFR3* mutations in bladder carcinogenesis, we assessed the incidence of *FGFR3* mutations in a large series of 132 bladder tumors of various stages and grades.

The high frequency of mutations that we found (48 of 132) confirmed that *FGFR3* mutations are a frequent event in bladder carcinomas. Five different mutations were identified, four of which (R248C, S249C, G372C, K652E) were detected in our previous series; the remaining mutation (Y375C) was identified for the first time in this series. This fifth mutation is also known to be involved in thanatophoric dysplasia. The S249C mutation (TCC → TGC) is the most frequent *FGFR3* mutation in bladder tumors (33 of 48, 69%). In contrast, this mutation was found in only 7 of 62 (11.5%) thanatophoric dysplasia cases, the most common mutation in this syndrome being R248C (CGC → TGC) (26 of 62, 42%; J Bonaventure, unpublished data). This probably reflects differences in etiology: many *FGFR3* mutations in bladder cancer could be caused by carcinogens whereas the germinal mutations in thanatophoric dysplasia are spontaneous mutations that preferentially create C-to-T transitions in CG dinucleotides.

We investigated the relationship between *FGFR3* mutations and tumor stage and found that the frequency of *FGFR3* mutations was very high in pTa tumors (74%). *FGFR3* mutations were present in only 21% of pT1 and 16% of pT2–4 tumors. No mutations were found in the 20 CIS examined. We performed laser microdissection on all of the CIS samples to exclude the possibility of contamination by normal DNA. *FGFR3* is therefore the first gene found to be preferentially mutated in pTa tumors. Unlike *FGFR3* mutations, loss of chromosome 9, the only other frequent genetic alteration found in pTa tumors, is also common in invasive tumors. *FGFR3* mutations were also strongly associated with low grade. No mutations were found in CIS, which were all grade 3 lesions. The percentage of *FGFR3* mutations in non-CIS G3 tumors was low for all stages examined, including Ta and T1 tumors.

Clinical evidence and molecular studies have suggested that there are two different pathways of bladder carcinogenesis, generating two different non-invasive bladder tumors: CIS, which often progress to pT1 and pT2–4 tumors, and pTa, which rarely progress. The highly significant difference in the frequency of *FGFR3* mutations between pTa tumors and CIS ($P < 0.0001$) provides additional evidence that these two noninvasive bladder cancers are different entities. Few studies have carried out a genetic analysis of CIS.[2,7] These studies have shown that CIS and invasive tumors have many genetic alterations in common, such as a high frequency of *p53* mutations and specific chromosomal losses not found in pTa tumors. The high frequency of FGFR3 mutations in pTa tumors, their absence in CIS and their low frequency in pT1 and pT2–4 tumors are consistent with the model of bladder tumor progression in which the most common precursor of invasive tumors is Cis (cis → pT1 → pT2–4) and not pTa tumors.[1–6] The mutated invasive tumors may arise from mutated pTa tumors that progress or from CIS that acquire FGFR3 mutations during progression to invasive tumors. There is also a third possibility that cannot be excluded: some mutated CIS may be the precursors of some mutated pT1 lesions. Studies on larger series and further detailed analyses of the genetic

profiles (chromosomal losses and amplifications and p53 mutations) of recurrent tumors from patients with progressive disease may help to distinguish between these three possibilities.

Somatic *FGFR3* mutations are probably tumor-specific, as no *FGFR3* mutations were found in 9 random urothelium biopsy samples of normal appearance from 9 patients known to have a *FGFR3* mutation in their papillary lesions (data not shown). The high frequency of FGFR3 mutations in pTa tumors indicates that FGFR3 activation is a key event in the development of these tumors. It would be interesting to analyze other molecules of the FGFR3 pathway, such as FGFR3 ligands or downstream signaling molecules, in pTa tumors without mutations in FGFR3.

pTa tumors are the commonest type of primary bladder tumor. These tumors rarely progress but recur in more than 50% of cases. Because most of these tumors carry FGFR3 mutations, the detection of such mutations in urine may provide an accurate additional means of follow-up and identification of tumor recurrences. This could be especially useful for low-grade lesions, which are difficult to detect by urine cytology and which harbor FGFR3 mutations in more than 80% of cases.

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